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Award Number: DAMD17-99-1-9172

TITLE: Functional Analysis of TGF-a/b Regulated Protein (TGFRP) in Breast Cancer Angiogenesis

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REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 Jul 99 - 30 Jun 02)

4. TITLE AND SUBTITLE
Functional Analysis of TGF-a/b Regulated Protein (TGFRP) in Breast Cancer Angiogenesis

5. FUNDING NUMBERS
DAMD17-99-1-9172

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Angiogenesis is critical for tumor growth, maintenance, and metastasis. Tumors with no vascular bed are small, necrotic, and incapable of intravasation and hematogenous transport. Therapeutic strategies targeting vascularization promise efficacy against all types of solid tumors, including breast cancers. Preliminary studies of the novel secreted protein TGFRP suggest that it is an important angiogenic factor, implicated in tumor vascularization, progression, and metastasis. The aim of this project is to elucidate the role of TGFRP in carcinogenesis by studying expression in normal development, inappropriate expression in tumors, and effects upon cultured endothelial cells. We have found the TGFRP is not expressed at the earliest stages of mouse development. However, using an in vitro assay system, we have found the TGFRP may act as a survival factor for endothelial cells. Completion of these studies will provide an understanding of the role of TGFRP in normal and misregulated vascular development, and may contribute to the development of novel therapeutics.

14. SUBJECT TERMS
breast cancer, angiogenesis, therapy, tumor progression

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The goal of these studies is to elucidate the roles of TGFRP (TGFα/β Regulated Protein) in breast cancer progression. We have been testing the hypothesis that TGFRP acts as an angiogenic factor in an autocrine and/or paracrine manner. Our findings thus far support this hypothesis. Elucidation of the angiogenic mechanism, of which TGFRP is likely a player, will reveal novel targets for breast cancer treatment which will in turn lead to new therapeutic strategies.

BODY

Task 1: Determine the expression pattern of *TGFRP*.

As previously reported, *TGFRP* is not expressed at detectable levels during early angiogenesis and vasculogenesis in mouse (embryonic day 6.5-11.5). To determine whether or not TGFRP has a role in later angiogenic/vasculogenic processes, we have examined expression in late gestation embryos (embryonic day 13.5-17.5) by *in situ* hybridization of paraffin sections. We have detected no specific signal above background levels with either antisense or sense control riboprobes. Several positive control riboprobes gave excellent results in these studies, giving us confidence that the protocol was working well. This suggests that TGFRP is either expressed at levels below the threshold of detection, or not all, during embryonic angiogenesis. Radioactive section *in situs* may prove to be more sensitive. Alternatively, TGFRP may play a role in homeostatic angiogenesis or vasculogenesis as might occur during wound healing or fracture repair (Gerber and Ferrara, 2000; Sherratt and Dallon, 2002). These studies are ongoing, although more effort has been channeled into furthering other tasks.

Task 2: Evaluate the role of TGFRP in angiogenesis

In vivo angiogenic studies of TGFRP as proposed originally have been postponed, as suitable resources are no longer available to us at Duke (see also previous report). We have therefore continued several lines of in vitro research. Our early results suggested that TGFRP may act on human microvascular endothelial cells (HMEC-1) in a non-autonomous manner. TGFRP acts as both a survival and morphogenetic factor (see earlier report). However, both human vascular endothelial cells (HUVEC) and HMEC-1 may be unsuitable for some angiogenic studies. HUVEC are derived from macrovascular source, while HMEC-1 cells have been immortalized

with large-T antigen, potentially altering their proliferative and survival responses (Pipas and Levine, 2001). We have therefore created a new cell line by immortalizing human microvascular endothelial cells (HMVEC) with the human telomerase catalytic subunit (hTERT). This method has been used successfully by others to immortalize cell lines (Salmon et al., 2000). Our cell line, dubbed IHMVEC, appears to retain characteristics of the primary line, and was used in subsequent proliferative studies.

We tested the mitogenic response of IHMVEC to TGFRP using a tritiated thymidine incorporation assay. These data demonstrate that TGFRP is not a mitogen for microvascular endothelial cells (Figure 1). These data do not refute the possibility that TGFRP is an angiogenic factor, but rather suggest that it may act in another manner. Two possibile functions include morphogenesis control and resistance to cellular stresses, such as hypoxia.

To test the first possibility, we performed long and short-term cell adhesion assays using TGFRP as an adhesive substrate. Although TGFRP does not promote adhesion after 90 minutes in culture (not shown), cell adhesion was significantly increased over BSA control after 24 hours (Figure 2). These data suggest that TGFRP may not act as a typical cell adhesion molecule, but rather through upregulation of other cell adhesion molecules or secretion of extracellular matrix components.

To test the second possibility that TGFRP promotes survival to cellular stresses, we mimicked hypoxia conditions through the addition of either $CoCl_2$ or DFO. Our data show that TGFRP-transfected MCF-7 cells survive in higher numbers that cells transfected with a control vector (Figure 3).

Task3: Identify and isolate the binding target of TGFRP

Isolation of the TGFRP receptor or binding target is necessary to fully characterize the signaling pathway activated by TGFRP. To that end we have successfully generated large quantities of high purity protein (as used above; Figure 4). Initial attempts at ^{125}I-labeling followed by binding assay were met with large background problems and other technical challenges, as described previously. To circumvent these problems we attempted to label the recombinant protein with biotin for the binding assay. Unfortunately, this method has proven not sensitive enough for binding detection. Thus, we have decided to proceed with the ^{125}I-labeling experiments, and are beginning to optimize the conditions.

While these experiments have been progressing we have also searched for downstream targets/effectors of TGFRP function. Intriguingly, we have found that

TGFRP may upregulate the expression of two receptor tyrosine kinases involved in angiogenesis: Flk-1, a receptor for VEGF, and Tie-2, a receptor for Ang-1 (Figure 5). These receptors may have roles both in de novo vessel formation as well as remodeling of existing vascular networks reviewed in (Liu et al., 2000). These data suggest that TGFRP may act in part by increasing the signal transduction through known angiogenic pathways.

KEY RESEARCH ACCOMPLISHMENTS

1. Determination that TGFRP is not expressed embryonically in the mouse.
2. Further support that TGFRP does not act directly as a mitogen for endothelial cells.
3. determination that TGFRP may increase cell adhesion, possibly by an indirect mechanism.
4. Determination that TGFRP may protect cells from hypoxia stress.
5. Determination that TGFRP may upregulate the expression of receptor tyrosine kinases important in known pathways of angiogenesis.

REPORTABLE OUTCOMES

All category of activities are in progress.

CONCLUSIONS

The studies summarized by this report provide support for the hypothesis that TGFRP promotes the growth and survival of breast tumors by promoting blood vessel morphogenesis, promoting other angiogenic signaling pathways, and protecting against the stresses of nutrient and oxygen depletion. While TGFRP has not been detected during mouse embryonic development, its expression may be tightly reguated at a level below the threshold of sensitivity of our analysis. Aside from this, it remains that TGFRP may be important for other homeostatic mechanisms in newborn and adult animals.

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APPENDIX

Four figures which are referenced in the body of this report.

APPENDIX

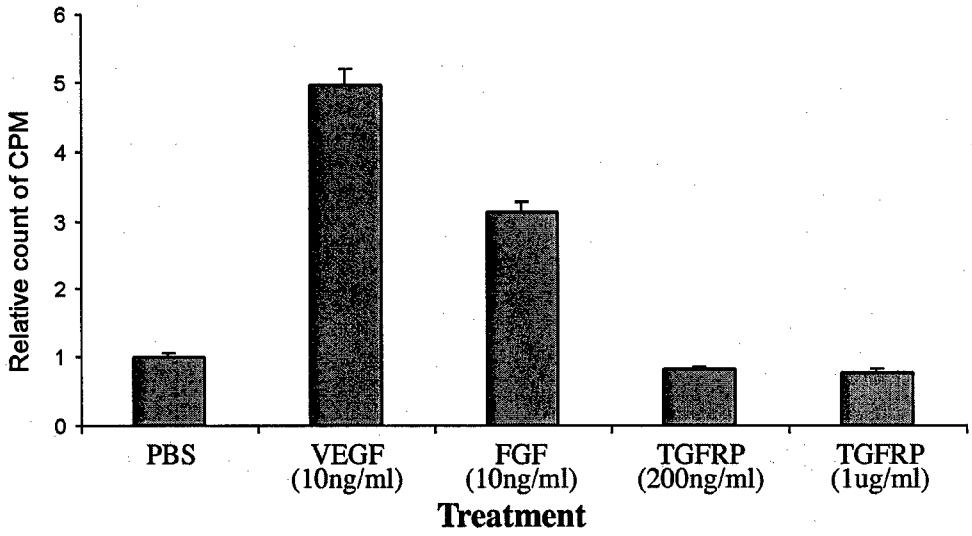

Figure 1. Tritiated thymidine incorporation assay. Serum-starved IHMVEC were treated with PBS, VEGF, FGF, or TPF at the indicated concentrations. Following 10 hours of culture, labeled thymidine was added and incubated for 4 additional hours. Cells were lysed, and labeled thymidine was quantitated by a scintillation counter.

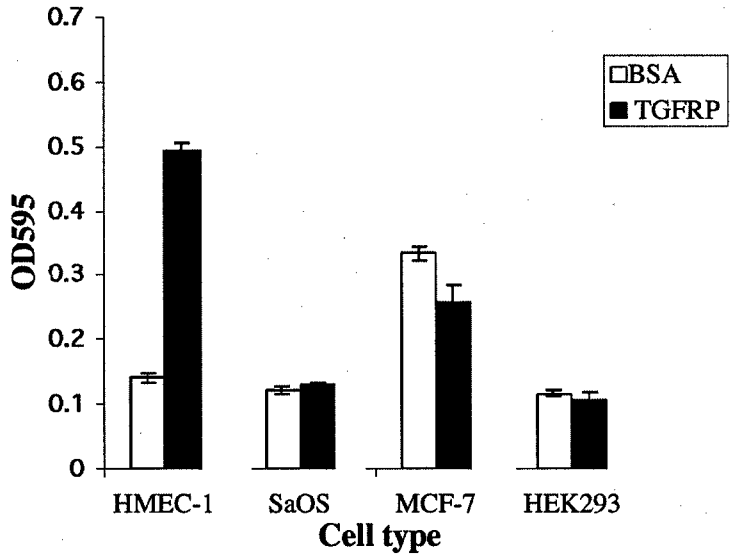

Figure 2. HMEC-1 long-term adhesion assay on TGFRP substrate. 96-well plates were coated with 10ug/ml TGFRP overnight at 4C, then blocked with BSA. HMEC-1 cells were plated at a density of 10^4 cells/well, and incubated for 24 hours. Wells were then washed with PBS and attached cells were stained with crystal violet. The remaining dye was quantitated using a plate reader.

APPENDIX

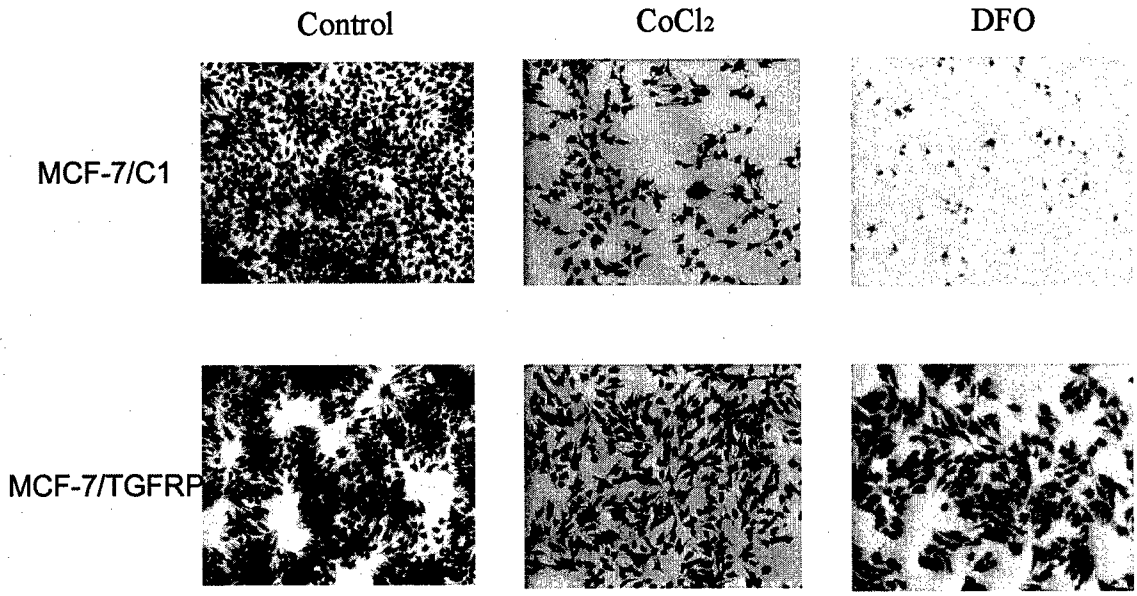

Figure 3. TGFRP-transfected MCF-7 cells are protected from hypoxia stress. Stable transfectants (MCF-7/TGFRP) and control cells (MCF-7/C1) were treated with either $CoCl_2$ or DFO for three days in serum-free conditions. Surviving cells were stained with DiffQuick.

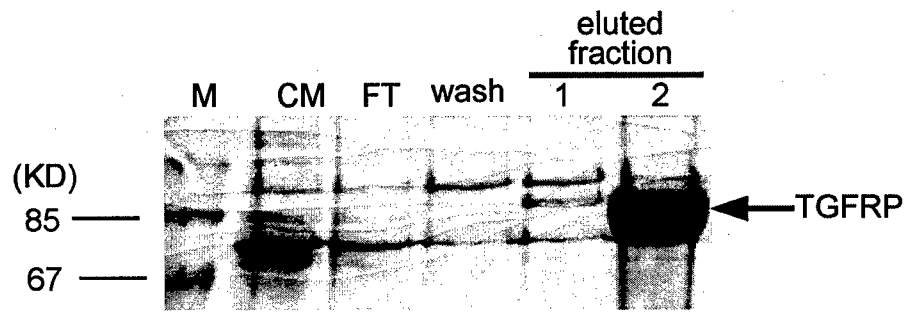

Figure 4. Purification of recombinant His(6)-tagged TGFRP from baculovirus. Conditioned media was loaded onto a nickel column (Qiagen), washed with 10mM imidazole, and eluted with 300mM imidazole

APPENDIX

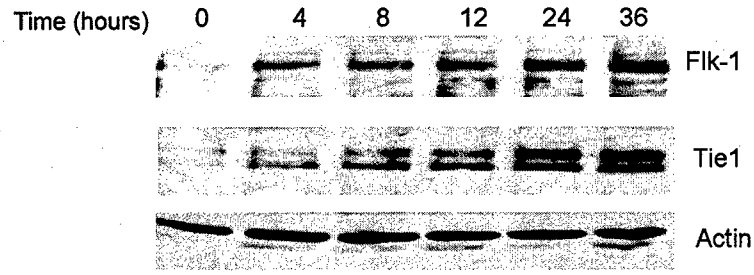

Figure 5. Induction of angiogenic receptors in IHMVEC by TGFRP. IHVEC were treated with TGFRP at a concentration of 200ng/ml. Cells were lysed, equally loaded into an SDS-PAGE gel, and transferred to a nitrocellulose membrane. Protein was detected with α-Flk-1, α-Tie-1, or α-actin antibodies as indicated.